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PHYTOCHEMICAL INVESTIGATION AND ANTIMICROBIAL ASSAY OF LEAVE EXTRACT OF *ZEHNERIA SCABRA*

THESIS SUBMITTED FOR PARTIAL FULFILLMENT OF THE REQUIREMENT OF MASTER OF SCIENCE (MSC) IN CHEMISTRY (ORGANIC)

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CERTIFICATION

This is to certify that the thesis entitled phytochemical investigation and antimicrobial assay on the leave extract of *zehneria scabra* submitted in partial fulfillment of the requirement of master of science (MSC) in organic chemistry, department of chemistry, University of Gondar, is a record of original research carried out by Habtamu Abebe, under my supervision, and no part of the thesis has been submitted for any other degree or diploma. The assistances received during the course of this investigation have been duly acknowledged. Therefore, I recommend that it is accepted as fulfilling the thesis requirements.

Kibur Hunie Tesfa (PhD) _____ _____

Name of advisor signature Date

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DMAPP	Dimethyl allyl pyrophosphate
IPP	Isopentenyl pyrophosphate

ABSTRACT

The genus zehneria have been under use since long time for the treatment of various diseases such as stomach pain, fever, skin disease, diarrhea, analgesic, bacteria and antibiotic properties. Zehneria Scabra is one of the medicinal plants used traditionally for the treatment of different disease. This study intended to identify the chemical constituents of the 99% CH_3OH leave extract of zehneria scabra. The phytochemical screening of methanol crude extract indicated the presence of alkaloid, flavonoid, terpenoids, glycosides, tannin, phenol and absence of carbohydrate and saponins. The crude extract fractions were tested against two bacterial species (S. Aureus and E.Coli) using agar well diffusion method at 100mg/ml concentrations in the presence of positive control. The chloroform and ethyl acetate fraction revealed more potent antibacterial activities against the growth of the bacterial strains than other solvent fraction of methanol crude extract. Isolation and purification of the crude extract was conducted by using preparative thin layer chromatography (10% ethylacetate:90% chloroform).The structure of the isolated component was determined by using a combination of spectroscopic techniques such as, IR, ^{1}H NMR, ^{13}CNMR and DEPT-135 Spectra.

Key words: phytochemical screening, antibacterial activity, bacterial species, zehneria Scabra, 5-(1', 2'-dihydroxy propan-2'-yl)-4, 6-dioxobicyclo [3.1.0] hex-2-ene-2-carbaldehyde.

1. INTRODUCTION

1.1. General

Since the beginning of civilization, people have used plants for different purposes in their daily lives. As such, plants and human beings are so intimately linked and discussion of human life on this planet would not be complete without a look at the role of plants [1].

Organic chemistry as stands today has developed largely from the chemistry of natural products. With the coming of modern spectroscopic techniques such as UV, IR, Mass Spectroscopy (MS), multidirectional NMR, and improved chromatographic techniques, a generation of new organic substances from terrestrial and marine organism are being discovered, many of which have biological activity [2]. Traditional medicine is the sum total of skills, knowledge and practices based on the theories, beliefs and experiences of indigenous to different cultures, that used to maintain health, as well as to prevent, diagnose, improve or treat physical and mental illnesses [3].

Plant is an important source of medicine and plays a key role in world health. Medicinal herbs or plants have been known to be an important potential source of therapeutics or curative aids. The use of medicinal plants has attained a commanding role in health system all over the world. This involves the use of medicinal plants not only for the treatment of diseases but also as potential material for maintaining good health and conditions. Many countries in the world, that is, two-third of the world's population depends on herbal medicine for primary health care. The reasons for this is because of their better cultural acceptability, better compatibility and adaptability with the human body and pose lesser side effects [4]. And also, plants with medicinal properties enjoy the highest consideration in indigenous systems of medicine throughout the world. For a developing country where resources and medicinal facilities are scarce, herbalists play a crucial role in the health care system for both humans and animals [5].

And also, Plants are being used as valuables sources of food and medicine for the prevention of various illnesses and the maintenance of human health. Medicinal plants are cheap and renewable sources of pharmacologically active substances and are known to produce certain

chemicals that are naturally toxic to microorganisms. The uses of medicinal plants as possible therapeutic measures have become a subject of scientific investigation [6].

Plants have been a direct provider of shelter, medicine, building material, fuel, water and foods for people and their livestock. Medicinal plants are those plants which are rich in secondary metabolites and are potential sources of drugs. They contain vitamins needed by human body for healthy living [7]. According to World Health Organization (WHO), more than 80% of the world's population relies on traditional medicines for their primary health care needs. The medicinal value of plants lies in some chemical substances that produce a definite physiological action on human body. The most important bioactive compounds in plants are alkaloids, flavonoids, tannins, terpenoids, glycosides and phenolic compounds. The phytochemical research based on ethno pharmacological information is generally considered an effective approach in the discovery of new anti-infectious agent from higher plants [6].

Demand for medicinal plant increasingly felt, in both developing and developed countries due to growing needs of natural products being non-toxic and benefit of side-effects, apart from availability at affordable prices. The medicinal plant sector has traditionally occupied a pivotal position in the socio cultural, spiritual and medicinal areas of rural and tribal families. To avoid the occurrence of toxic side effects in a long-term usage of synthetic drugs during treatment of chronic diseases, herbal drugs are being used widely. Therefore proper documentation of traditional knowledge is needed [8].

Ethno botany is the scientific study of the relationships between plants and people. It was stated that Ethno botany appears to be a promising discipline that can play a key role as a mediator of dialogue between different academic disciplines and traditional knowledge, a union essential to enable contextualized and sustainable alternatives to exploitive practices and biodiversity management. Hence, ethno botanical studies play significant roles in contributing techniques of community based resource management and conservation. This is because the science of ethno botany is an endeavor which attracts people from various academic disciplines. Ethno botanists and local people face the challenging task of not only recording knowledge of the plant world but also applying the results of their studies to biodiversity conservation, community development and primary healthcare services involving medicinal plants [9].

In Ethiopia, traditional medical practioners use different categories of medicine such as plants, animal products and minerals. The drugs are prepared in various dosage forms such as liquid, powder and prescribed in a non-formulated form [10]. The use of traditional medicinal plants has been widely practiced in Ethiopia. Indigenous peoples of different localities in the country have developed their own specific knowledge to use, manage and conserve plant resources, which gave traditional medicine its diverse nature. Extensive use of traditional medicine in Ethiopia could be accredited to efficacy against certain type of ailments, economic affordability, physical accessibility, and cultural acceptability as compared to modern medicine. Medicinal plants contain drugs used for suppressing, preventing or curing many forms of diseases, and more than 95% of traditional medical preparations are of plant origin in Ethiopia [11].

Ethiopia is endowed with diverse vegetation types ranging from high altitude Afroalpine vegetation in the central highlands to arid lowlands in the East, and rainforests in the West. The altitude of Ethiopia ranges from 125 m above sea level to 4533 m above sea level. And it possesses more land above 2000 m than any other country in Africa. The highlands that host most of the Afromountain vegetation are divided into the Western and Eastern highlands by the East African Rift Valley. The country has the fifth largest flora in Africa and tremendous floristic diversity; with an estimated 6,500-7,000 species of higher plants of which about 12% are endemic. It is there for not surprising that some of these plants have chemical compounds of therapeutic value that may be used in the treatment of major diseases such as HIV/AIDS, malaria, cancer, analgesic, etc [12].

The history of plant use by humans for the treatment of various diseases is as old as the history of the human species. Humans had been looking to nature to provide them with remedies for their health problems most of which are derived from plant products. Hence, plants have been used as a source of medicine in developed and developing countries in general and in Ethiopia in particular since time immemorial [9].

The introduction of modern medicine to Ethiopia dates back to the 16th century during the regime of Emperor LibneDingel (1508-1540). The first government run modern healthcare was established in 1906 with the opening of Menelik II Hospital in Addis Ababa. However, the growth and development of modern health care in Ethiopia as a whole has been very stunted and

to date, its coverage is less than 50% of the population. The vast majority of the rural populations, therefore, still depend on traditional medicine and its practitioners. The use of traditional medicine is still widespread in Ethiopia, and its acceptability, availability and popularity is no doubt as about 90% of the populations use it for health care needs. In Africa up to 80% of the population uses traditional medicine to help meet their health care needs [13].

Due to inefficiencies or lack of hospitals and social services in rural area, many people are currently resorting to traditional medicine for primary health care and also, due to high costs, inaccessibility, cultural incompatibility, and self-reliance among others. They also employ herbal medicines because of cultural preferences and perceived effectiveness [14]. Ethiopia is also a home of many languages, cultures and beliefs which in turn have contributed to the high diversity of traditional knowledge and practices of the people which, among others, include the use of medicinal plants. Plants have been used as a source of medicine in Ethiopia from time immemorial to treat different ailments [13]. Therefore scientific evaluation of these plants may provide modern medicine with lead compounds for the development of new drugs.

1.2. Statement of the Problem

Demands of traditional herbal medicines are increasing day by day not only in the developing countries but also in the developed countries throughout the world. The demand is due to the increased traditional herbal medicines and acceptance of Ayurveda, due to their safe therapeutic effect. Such, modern people rely more on drug resources of plant origin. The world scientific research is getting momentum to evaluate the medicinal properties and pharmacological activities of *zehneria* species. Based on various experimental researches, numerous pharmacological activities or medicinal properties of *zehneria* species have been reported [15]. The review articles and scientific studies on *zehneria* species suggested an enormous biological potential of these plants. Pharmacological, medicinal and traditional studies with standardized extracts and isolated constituents need to be performed in order to investigate unexploited potential of this plant. In different countries the use of *zehneria* species in various ways would create attention about this plant for their medicinal, pharmacological and traditional values. Although this herbal medicine has extensive therapeutic effect, no sufficient study has been

conducted. Even existing studies have focused on other parts of the plant without paying attention to the leaves. In this study medicinal, pharmacological and structural aspect of leave extract of *zehneria scabra* was examined.

1.3. Significance of the Study

This study investigates the relevance of *zehneria scabra* leaves to human health. Most scientific journals didn't cover the important of *zehneria scabra* leaves to health. Thus the resurfacing of the role of this traditional medicine is a core role of this study. Also, this study will help raise the status of the *zehneria scabra* medicinal values by extensively elaborating its components, utilities and curative effects.

2. OBJECTIVES OF THE STUDY

2.1. General Objectives

The objective of the study was to extract, isolate and characterize the secondary metabolites and determine antimicrobial assay from the leaves of *zehneria scabra*, family *Cucurbitaceae*, using 99% methanol ($HOCH_{3)}$ as solvent.

2.2. Specific Objectives

- ✓ To isolate the major constituents of the leaves of *zehneria scabra*, family *cucurbitaceae* with the help of chromatographic techniques.
- ✓ To characterize (elucidate) the structure of the isolated compounds by spectroscopic techniques (IR, ^{1}H NMR, 13C NMR and DEPT-135 Spectra).
- ✓ To test bioactivity on pathogenic bacterial species (E.Coli and S. Aureus) using methanol crude extract fraction.
- ✓ To test phytochemical constituents of methanol crude extract of *zehneria scabra* leaves.

3. LITERATURE REVIEW

3.1.The Genus of *Zehneria*

The genus *zehneria* is climber belongs to the family of *cucurbitaceae*. The *cucurbitaceae* is commonly known as *cucurbits* and is most diverse in tropical and sub-tropical regions with hot spots in South East Asia, Madascar, Africa, and Mexico. In general, members of *cucurbitaceae* (*pumpkin, lufas, zucchini, watermelons, cucumbers, courgettes,* and *summer squash)* are edible and found in all continents of the world. In African continent, this family is represented by 24 genera and 54 species. The wild genera are *langeneria*, *luffa*, *momordica* and *zehneria*. The family is economically very important being the major source of food and forage and its great diversity (ranked as fifth largest family in flowering plants) has also attracted much interest in ecological as well as systematic studies. All members of the *Cucurbitaceae* family have alianous structure of the plant body, the development of characteristic fleshy fruits (referred to as pepo) and a similar mode of sex determination. The *Cucurbitaceae* are mostly prostrate or climbing herbaceous annuals or perennials, variably pubescent and sometimes with tuberous rootstock. They are further characterized anatomically by commonly having angled stems with bicollateral vascular bundles often arranged in two concentric rings [16].

The *Cucurbitaceae* family is well represented in the paleotropics, and includes several genera widely distributed across many environments. Species in the genus *Zehneria* as formerly taxonomically circumscribed are found across the paleotropics, with several species in Africa, and mainland and insular Asia. *Zehneria* in that sense is species-rich in East Africa, but most diverse in South East Asia. *Zehneria* was first described by Endlicher based on collections made by Bauer on Norfolk Island in 1804–1805. And also, Cogniaux considered *Zehneria* to be part of the genus *Melothria* L., a large assemblage with many species in the old and new Worlds. The definition of *Melothria* was broadly constrained to cucurbitaceous plants sharing a common ancestor and floral type with a pantropical distribution [17].

The genus *zehneria* was divided into two sub genera by Jeffrey in 1962. The sub genus *zehneria* have long filaments, arcuatethecae, and finely papillose filament hairs; and sub genus

pseudokedrostis (Harms) C. Jeffrey has sub-sessile ancestors, straight thecae, and coarsely papillose filament hairs [18]. *Curcubutaceae* families are widely used in African communities as containers and utensils. The cut stems of *Zehneria scabra* sond and squeeze the sap onto fresh wounds to enhance healing. Among the Ogiek, *Lagenaria sphaerica*, naudin is externally used for skin conditions ranging from scabies and leprosy to candiasis. Elsewhere, extracts from the family have been used to treat tumors. There are also instances where the *Curcubitaceae* have been reported as being highly toxic to humans and to higher animals. Management of diabetes in Ayuverdic medicine is achieved through the use of *Momordica charantia* L. Other members of this genus, e.g., *Momordica reticulate* Salisb, are capable of accumulating selenium which is fatally toxic [19].

3.2. Description and Uses of *Zehneria Scabra*

Zehneria scabra "Local Name, Hareg Ressa" is one of the families of *cucurbitaceae* which has wide traditional uses. *Zehneria scabra* attracts greater attention because they have high medicinal value and due to herbal folklore practices however, lack of adequate information on the nature of bioactive principles and its therapeutic action [20]. *Zehneria scabra* is a perennial herb, climber or tailing to 6 m, it mostly found in tropical and southern Africa. It is mentioned in Ethiopian folk medicine for the chemotherapy of various infectious diseases and enormous ethno-botanical value, as used by tribes for various treatments such as tribal people used the root of *Zehneria scabra* to hang in front of their house believing that it will prevent the entry of disease causing pathogens and also, used with milk for the treatment of fever and diarrhea [19]. The leaves of *zehneria scabra* used traditionally for the treatment of malaria, inflammation, pain, bacteria and parasitemia management. It acts as an important medicine for livestock in various ailments and fruits of *zehneria scabra* are reported to cure stomachache [21].

Figure 1. The typical leaves of zehneria scabra (photo taken by the researcher himself in February, 2017).

3.3.The Taxonomic Position of *Zehneria Scabra*

Zehneria scabra, Renner and Pandey (2013).

Kingdom: Plantae

Order: *Cucurbitals*

Family: *Cucurbitaceae*

Sub-family: *Benincaseae*

Genus: *Zehneria*

Species: *Scabra*

3.4. Ethno-Botanical Uses of the Genus *Zehneria*

Several species of zehneria have important medicinal properties. In African countries, villagers generally consume leaves, fruits and flowers of cultivated *cucurbits* and also harvest leaves and fruits of some wild *cucurbits* for consumption and medicinal use. *Zehneria* species have enormous ethno-botanical value and are used by different tribes for food as wild edible plants and treatment of various ailments. The root extract of *Z. scabra* is used with milk to treat fever and diarrhea while the leaf extract is used to treat skin rashes and has anti-bacterial and anti-inflammatory properties. Leaves of *Z. scabra* and bark are pounded and rolled in cloth, and tied on swelling to reduce the effect [15].

3.5. *Zehneria Scabra* (Local Name: Hareg Ressa)

According to Stace, the anatomical characterization of these plants is not affected by environmental changes. Anatomical knowledge has been utilized to delimit species, genera and families in plants. It is widely used in systematic identification, placing anomalous groups in a satisfactory position in classification and explaining patterns of relationship that may have not been clearly expressed in morphological features [22].

Zehneria species belongs to the family of *cucurbitaceae* and is well known for its therapeutic properties in the folk medicine of many countries [19]. *Zehneria* species is being widely used traditionally to treat various diseases; some species have shown antibacterial activity against E.Coli and Pseudomonas aeroginosa, antimalarial activity, skin rashes, diabetes and etc [23].

A literature survey on the chemical constituents of the genus *zehneria* revealed that the presence of alkaloids, amino acids, flavonoids, triterpenoids and phenols. These types of metabolites have been isolated from leaves, root and flower of the genus *zehneria*[19]. There are many different classes of naturally occurring compounds. One of the major components of the genus *zehneria* is terpenoids. Terpenoids are one of the subgroup of naturally occurring compounds which occur in plants, a few of them have also been obtained from other sources. Terpenoids are volatile substances which give plants and flowers their fragrance. They occur widely in the leaves and

fruits of higher plants, conifers, citrus and eucalyptus. The term 'terpene' was given to the compounds isolated from terpentine, a volatile liquid isolated from pine trees. The simpler mono and sesquiterpenoid is chief constituent of the essential oils obtained from sap and tissues of certain plant and trees. Monoterpenoid are common in many plant species and are used in cosmetic, non-cosmetic and pharmacological preparations, as well as in food industry and also exhibit effect of relaxation on ileum smooth muscle [24].

Due to wide range of biological activity, terpenoids have extensive applications in the fields of pharmaceuticals, cosmetics, colorants, disinfectants, fragrances, flavorings and agrochemicals. And several terpenoids have also been used as drugs to benefit human health, such as artemisinin used as an antimalarial drug. Paclitaxel, known as taxol, is an effective anti-cancer agent. Avicins and parthenolide have been shown to reduce growth of tumor cells. Betulinic acid was found to exhibit anti-HIV-1 activity. Carotenoids, such as lycopene and astaxanthin, are the focus of research into their potential benefits for human health and treatment of disease, despite their diversity in structure and function.All terpenoids are made from the same five-carbon building blocks, isopentenyldiphosphate (IDP) and dimethylallyldiphoshate (DMADP). IDP and DMADP in turn originate from either of two distinct pathways [25]. A group of terpenoid that is attracting attention lately is pentacyclic tri-terpenoids (PCTs). PCTs are a class of C30 terpenoid compounds which occur widely in plants [26].

Triterpenoids constitute a wide, biologically interesting group of terpenoids and include a large structural diversity of secondary metabolites with more than 100 carbon skeletons identified from terrestrial and marine living organisms. This class of natural products, including triterpenes, steroids, limonoids, quassinoids, and triterpenoidal and steroidal saponins, consists of over 30,000 compounds isolated and identified. Most of triterpenic skeletons are tetracycles, containing three six-membered and one five-membered ring, and pentacycles, either with four sixmemberedand one five-membered ring or five six-membered rings. However, acyclic, mono-, di-, tri-, and hexacyclic scaffolds have also been isolated and identified from natural sources [27].

The terms triterpenes and triterpenoids are often used to describe the same C30-terpene compound. However, they need to be differentiated based upon their occurrence, biosynthesis and biotransformation products. The term 'triterpene' is used to describe naturally occurring terpenes whereas; the broader expression 'triterpenoid' includes natural degradation products. Triterpenes are originally synthesized by plants as metabolites, and are abundantly present in the plant kingdom in the form of free acids or aglycones. Still today, at least 80 distinct types of both the structure and the chemical characteristics of triterpenes have been shown. It is well-recognized that triterpenes have long been used as flavors, pigments, polymers, fibers, glues, and waxes. In many Asian countries, herbal products containing triterpenes are widely prescribed to prevent or treat a variety of diseases by the traditional healers [28].

The term triterpene refers to three monoterpenes and consequently to 30 carbons grouped in six isoprenyl units. Depending on the plant species, secondary metabolites belonging to this family are mostly stocked in the mitochondria, microsomes, or chloroplasts of cells. These components and their glycosylated homologs play crucial roles in protecting the plant against insects, fungi, and bacteria. Moreover, many neither tetra nor triterpenes derived from apotirucallane skeletons, by losing an isobutyl moiety, have proved their anti-feeding and anti-herbivore activities. Steroids possess a fully or partially reduced cyclopentaphenanthrene scaffold, sometimes bearing methyl groups at C-10 and C-13. However, the backbone of the side chain at C-17, its length, and the stereochemistry of some of its chiral centers lead to different steroid skeletons. Since the discovery of limonin in 1960, many skeletons of natural limonoids have been characterized. Steriod is compound with cyclopentanonehydrophenanthrene ring ($C_{17}H_{28}$). Sterol widely exists as free form, esters of fatty acid and glycoside with wide distribution among animals and plants. The main sterol found in animals is represented by sterol C_{27}while β-sit sterol, stigma sterol and campe sterol are commonly found in plants [27].

Some of the representative structure of triterpenoids is as shown below.

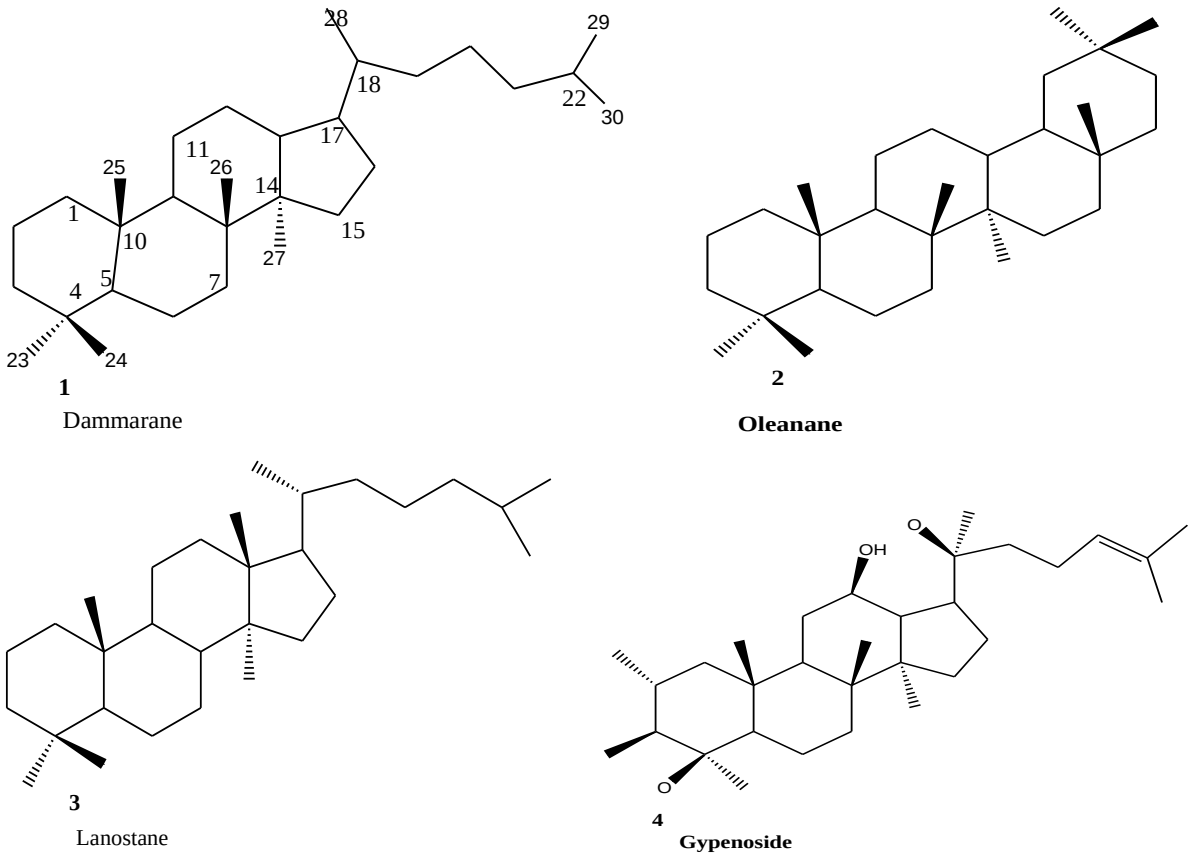
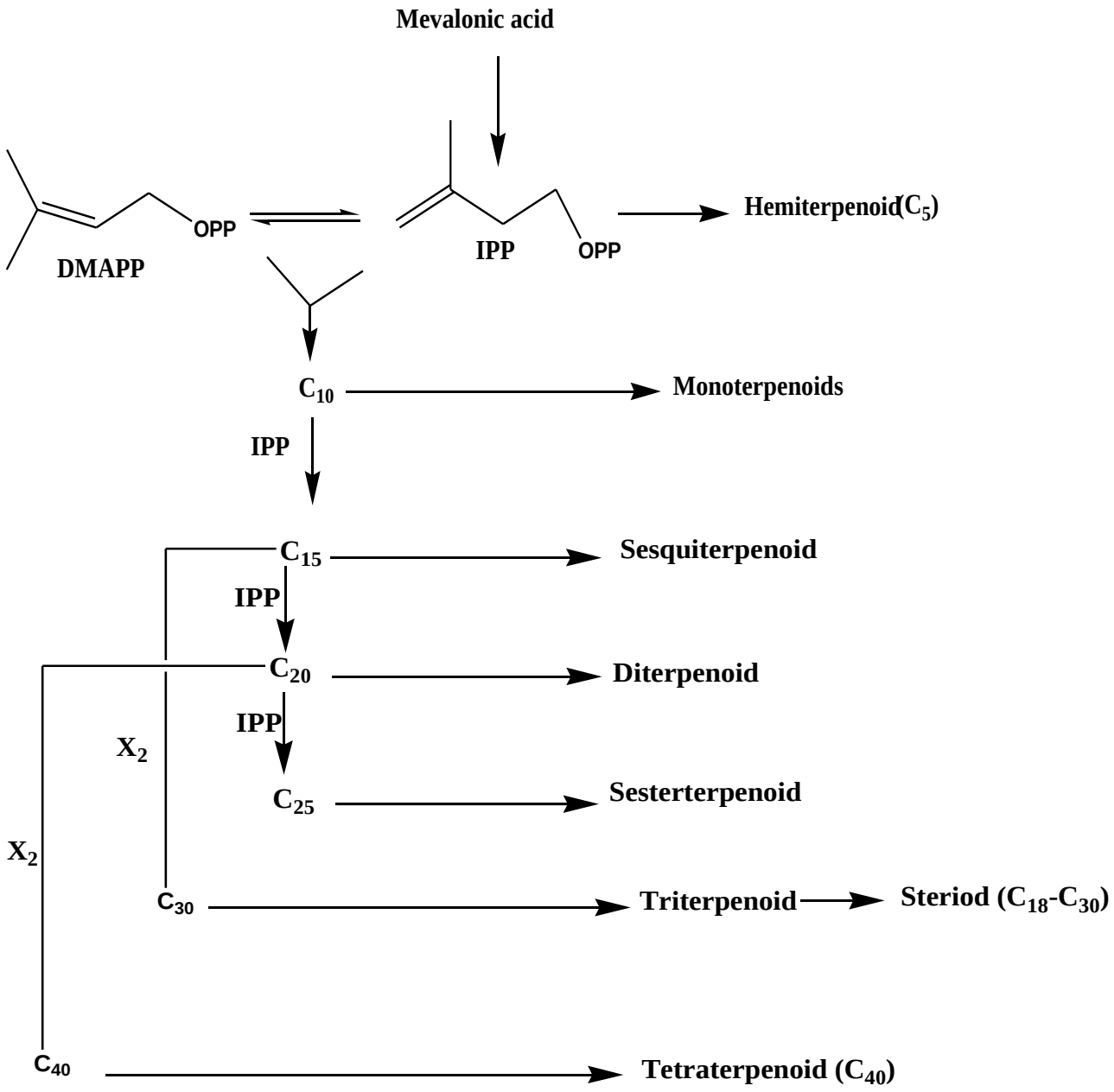


Figure 2. Structure of triterpenoids from various species

3.6. Biosynthesis of Terpenoids and Synthetic Pathway

The synthetic pathway is as shown below.

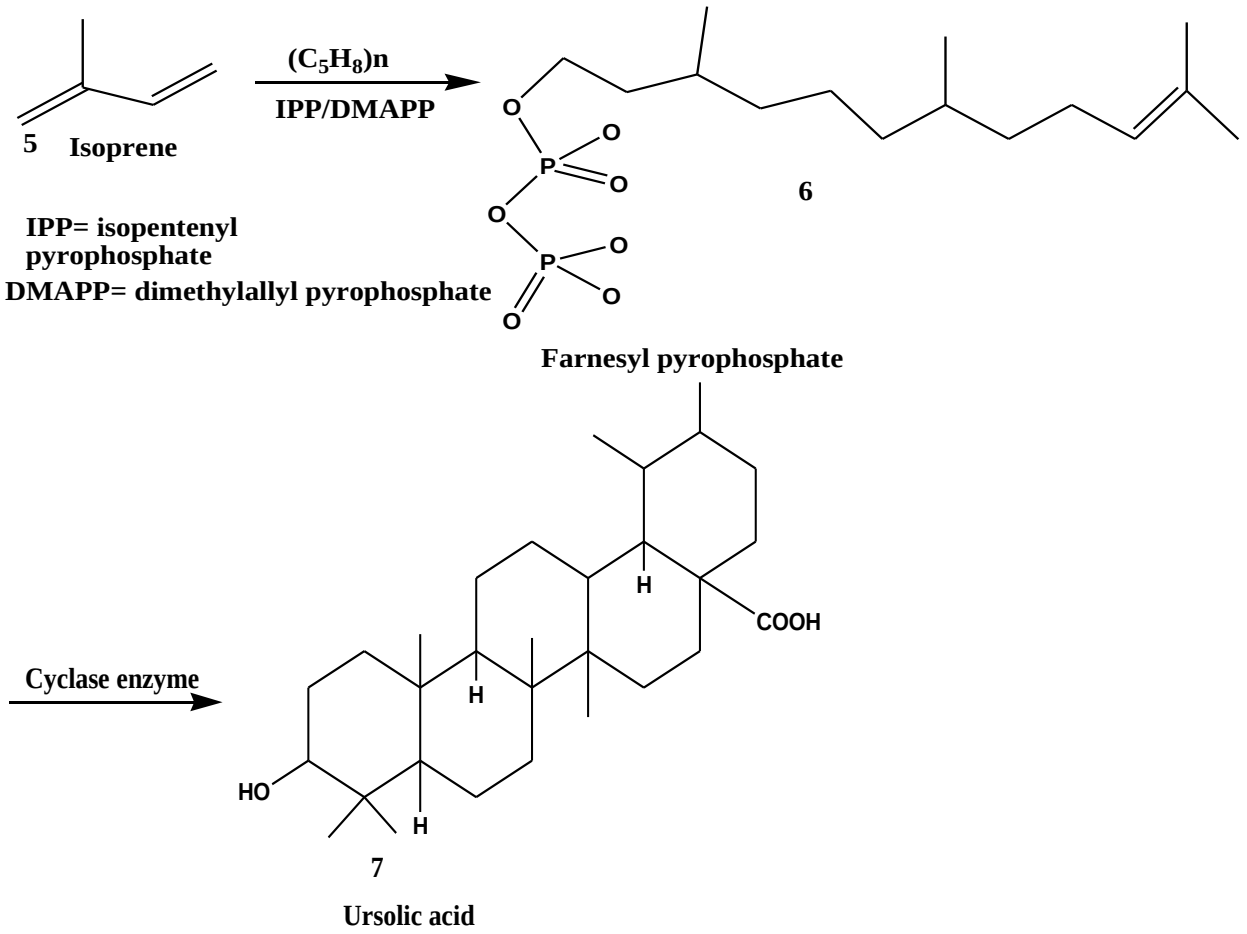


Scheme 1. Biosynthesis of Terpenoids

3.7. Source and Synthesis of Tri-terpenoids

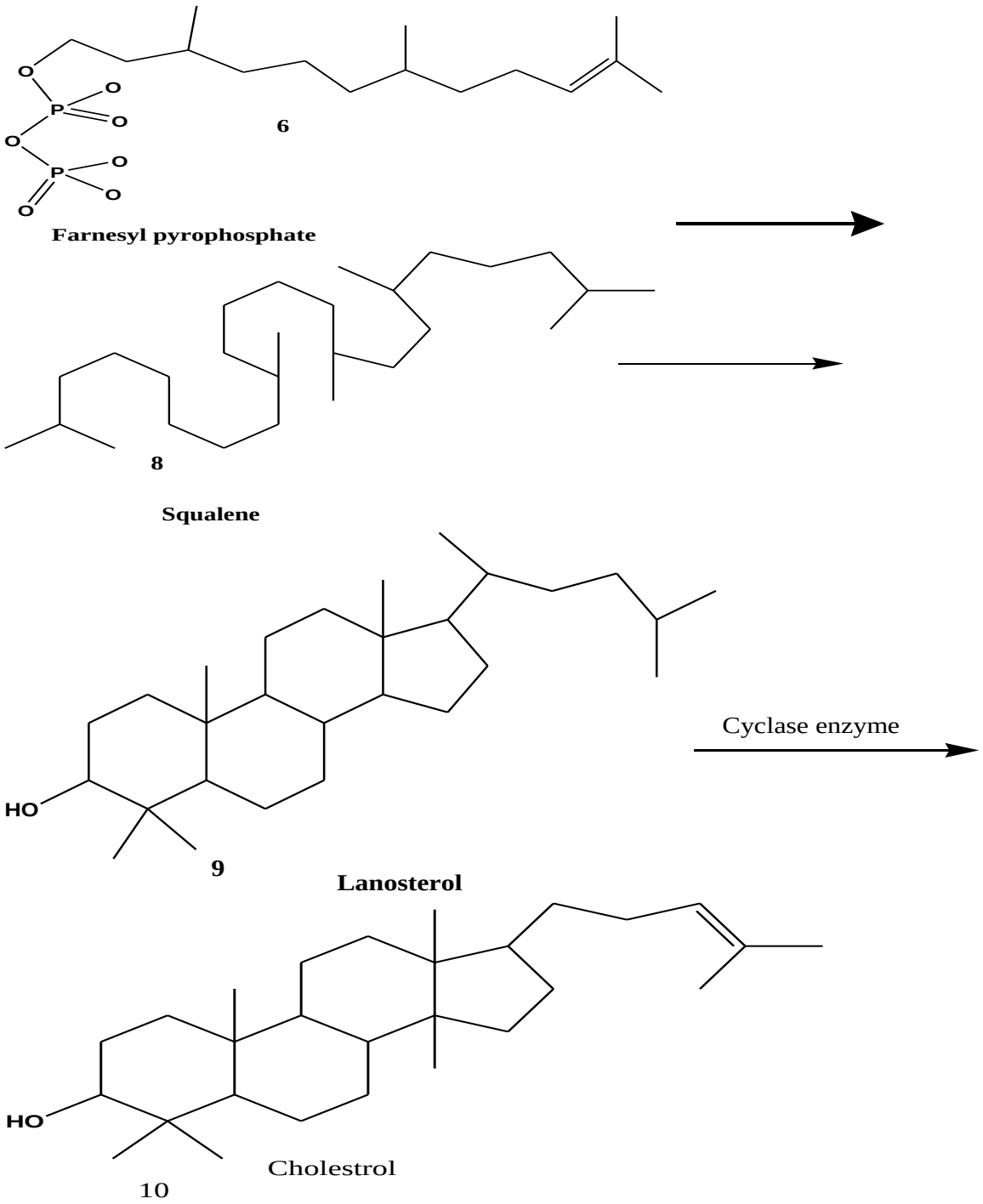
Triterpenoids are synthesized from isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP). For this cyclization, three prenyltransferases synthesize the linear prenyl pyrophosphates geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP). Squalene is in turn derived biosynthetically by the cyclization of a number of units of isoprene, $(C_5H_8)_n$ which undergo folding through 20 different patterns in the presence of prenylpyrophosphates to form monocyclic, dicyclic, tricyclic, tetracyclic, or pentacyclic derivatives. A family of oxidosqualenecyclases may produce only a single product, such as lupeol cyclases, but there are also multifunctional oxidosqualenecyclases that use dammarenyl cation intermediates to produce many products. Once squalene undergoes cyclization, it goes through the cytosolic mevalonate pathway to make a proximate tetracyclic C30 compound, lanosterol, which further undergoes oxidation and catabolic metabolism to form cholesterol [27].

Different patterns of cyclization of squalene to form triterpenoids. (IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate) and some of triterpenoids synthesis are as shown below.



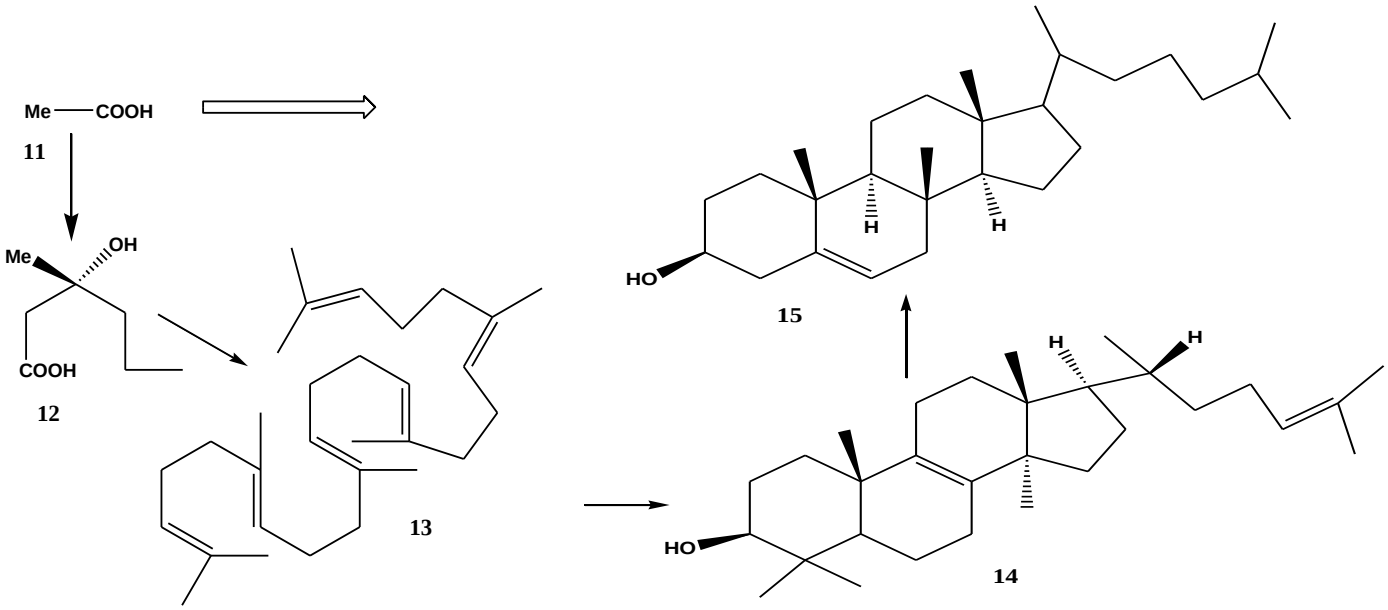
Scheme 2. Synthesis of triterpenoids from isoprene units

Synthesis of triterpenoids by cyclization of squaline



Scheme 3. Differenet patterns of cyclization of squalene to form triterpenoids

Synthesis of triterpenoids in mevalonate pathway and the synthesis is as shown below.



Scheme 4. Synthesis of triterpenoids in mevalonate path way

3.8. Previous Chemical Studies on *Zehneria Scabra*

Previous phytochemical studies on *zehneria scabra* revealed that the presence of flavonoids, alkaloids, terpenoids, phenols, glycosides, and tannins [20]. Gypenoside is other compounds isolated and characterized from the roots of zehneria scabra [29]. Due to presence of many secondary metabolites in *zehneria scabra* used in traditional medicine with diverse biological activities, for example, skin rashes, antimalarial, infectious disease, stomach pain, treat livestock, diarrhea and etc [23].

For the first time [29] isolated Gypenoside from the roots of zehneria scabra and the chemical structures of Gypenoside is as shown below.

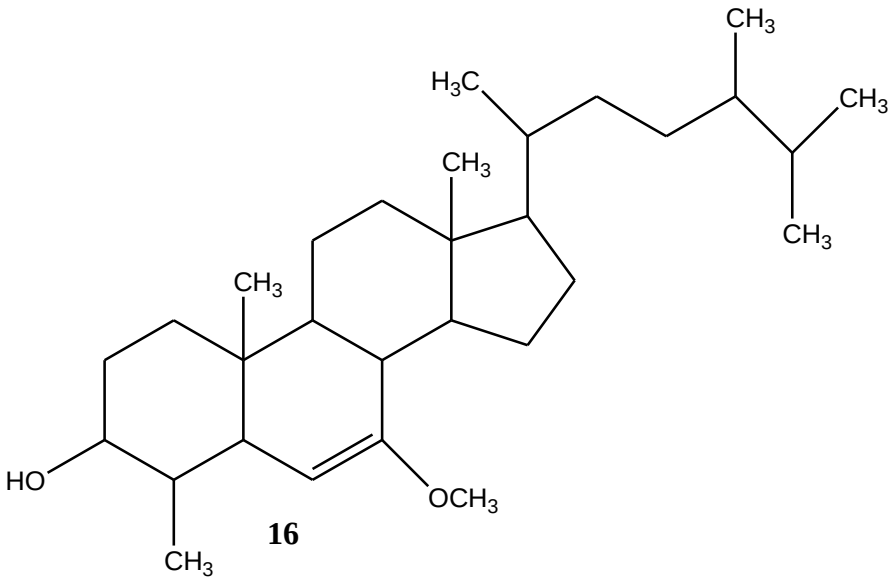


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Figure 3. Structure of Gypenoside isolated from the roots of *zehneria scabra.*

4. EXPERIMENTALSECTION

4.1. General Experimental Procedure

A UV-Vis spectrum was measured using a GENESY'S spectrometer (200 – 800 nm) in methanol at room temperature. Infrared spectrum was recorded using KBr pellets on Perk-Elmer BX Infrared Spectrometer in the range 4000-400 cm^{-1}. Nuclear Magnetic Resonance (NMR) analysis was recorded on a BrukerAvance 400 MHz spectrometer with deutrated CD_3COCD_3 using Tetramethylsilane (TMS) as internal standard. Structural elucidations were conducted on the basis of 1D NMR Spectra (^{1}H NMR, ^{13}C-NMR and DEPT-135). Analytical thin layer chromatography (TLC) was carried out with pre-coated 0.2mm silica gel 60 F_{254} on aluminum foil and compounds on TLC were detected under UV lamp at 254 and 365nm. The crude extract was fractions by different solvent system and bioactive component was isolated and purified by using preparative thin layer chromatography in a chosen solvent system.

4.2. Chemicals and Materials Used

PTLC chamber, rotary evaporator, electronic grinder, mortal and pistil, mechanical balance, flask, beaker, UV lamp, pippete, laboratory glass wares, hot plate, test tube, capillary tube, evaporating dish, separator funnel, filter paper, petridish, Mueller Hinton agar, 99% methanol, n-hexane, petroleum ether, ethyl acetate, chloroform, acetone, iodine crystal, *zehneria scabra* leaves and vancomycin were used.

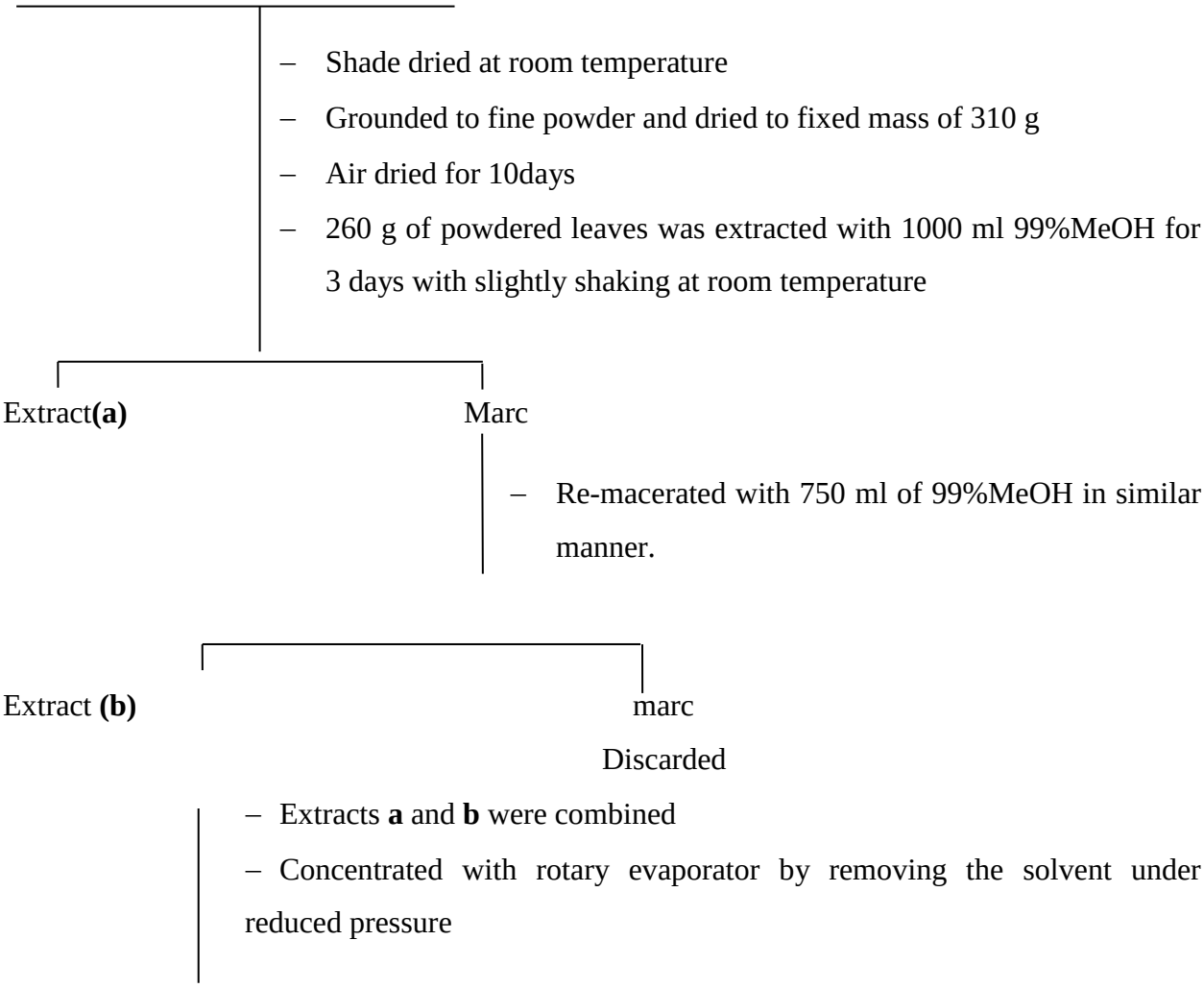
4.3. Plant Material Collection

Fresh *zehneria scabra* leaves were collected in polythene bags in April, 2017 from Azezo (Gondar, Amhara National Regional State, AR) located 10 km away from Gondar city, on the way to Addis Ababa. The collected plant sample was identified and authenticated by the botanist Mr Banteamlak Habtamu department of biology at Gondar University. The leaves were thoroughly washed, mixed and spread to dry at room temperature in the chemistry laboratory for about 10 days and then chopped in to smaller size to enhance drying. The dried leaves of *zehneria scabra* were deposited in organic chemistry laboratory of the University of Gondar.

4.4. Extraction and Isolation

The leaves of *zehneria scabra* dried to constant weight of 310g, reduced to coarse powder and then in to fine powder using electrical grinder (Model: IKA-WERKE GMBH AND CO.KG). The crude extraction was carried out by maceration protocol. Briefly, two hundred sixth gram (260g)of coarsely powdered leaves of *zehneria scabra* was weighed and soaked in 6000ml flask containing 1000ml 0f 99% methanol for 72 hrs with slightly shaking three times a day at room temperature. The extract was filtered with whatman filter paper No.1. The marc left was further extracted with the same solvent for 72 hrs in similar manner, for a total of 6 days in order to obtain a better yield. After filtration, the two extracted solutions were combined and concentrated using a rotary evaporator (Model: RE200) on water bath at a temperature of 40-45 ^{0}c which produces blue green crude extract (29g, 11.15%). Then, the concentrated filtrate was frozen in a deep freezer until required for further experiments. All the chemicals and reagents used for extraction and isolation were from organic chemistry laboratory and of analytical grade.

Scheme: Extraction of the Plant Material



Scheme 5. Plant material extraction procedure

The methanol crude extract was subjected to partition or separation by using separator funnel with petroleum ether, chloroform, ethyl acetate and methanol and each fraction was monitored by TLC with increasing polarity of ethyl acetate and chloroform in n-hexane.

Each solvent fraction of crude extract is given in the following figure

Petroleum ether fraction Chloroform fraction

Ethyl acetate fraction Methanol fraction

Figure 4. Methanol crude extract fractions

All solvent fractions were collected in different flask and concentrated in rotary under reduced pressure and all crude fractions were kept in deep refrigerator until required for further experiments and antimicrobial activity study.

From the four fractions, the chloroform fraction in the eluent system chloroform: ethyl acetate (9:1) gives three separate spots in TLC and the spots are shown in Fig. 5.

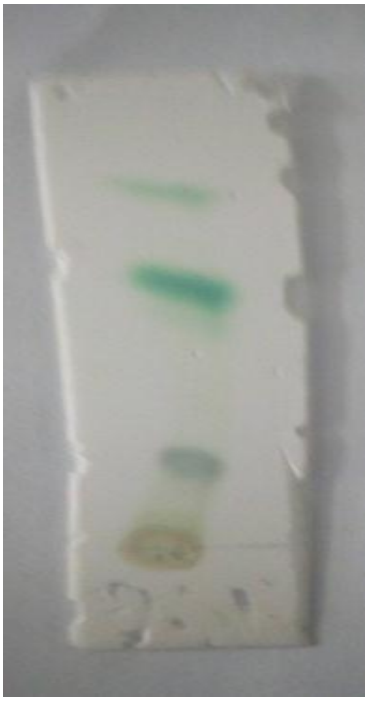


Figure 5. The TLC spots of chloroform fraction of crude extract

By using chloroform: ethyl acetate (9:1) eluent system, the chloroform fraction was subjected to preparative thin layer chromatography and gave separate colored compounds. The colored compounds were shown in the following figure. However, all compounds were not visible with necked eyes and some of them are visible under UV lamp at 254 nm and 365 nm.

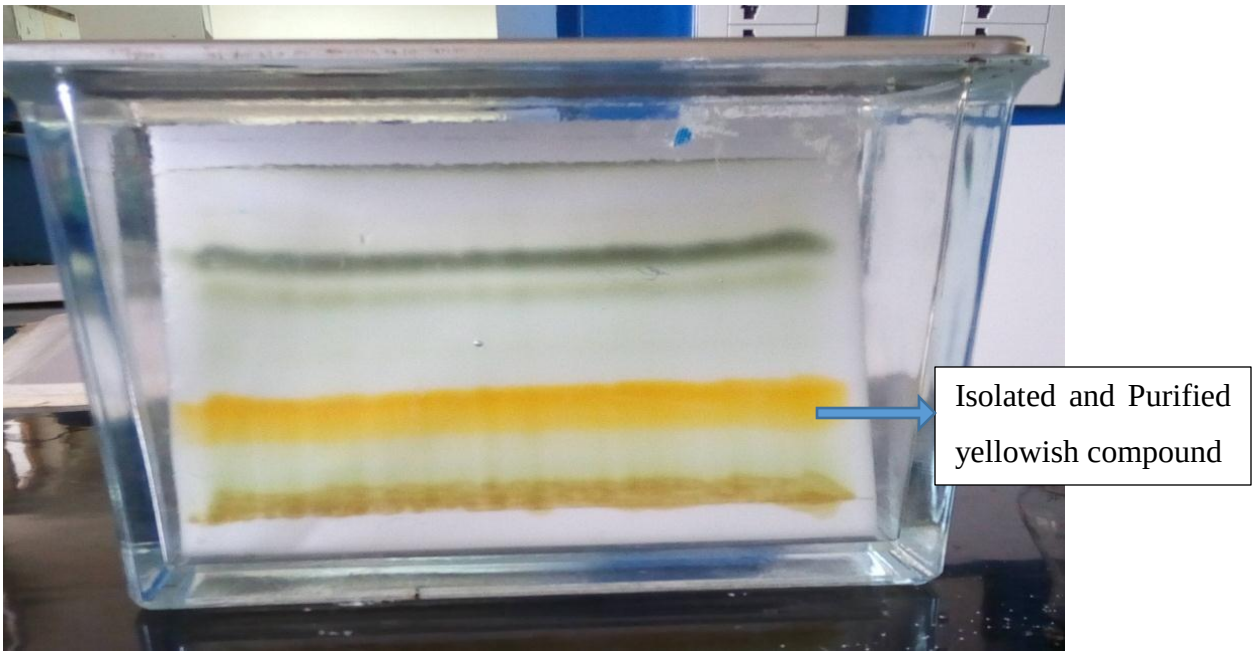


Figure 6. Preparative TLC profile for chloroform fraction in chloroform: ethyl acetate (9:1) eluent system

The six different colored compounds were separated and each one of them was collected in 50 ml flask. Then, each collected sample was dissolved in acetone and filtered with whatmann filter paper No.1.

For further information the six colored compounds were shown in the following figure.

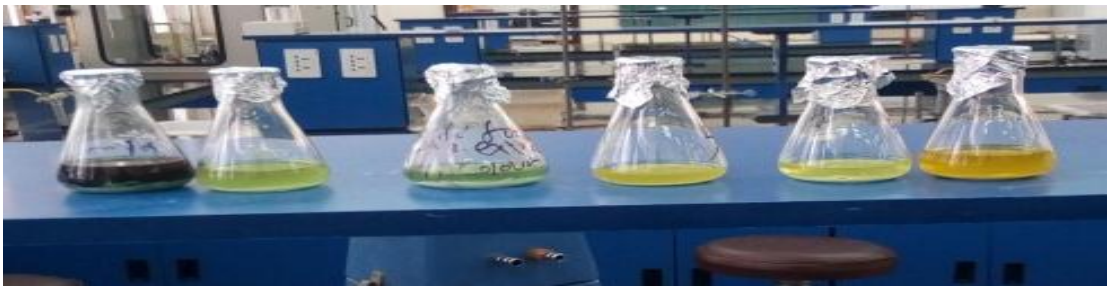


Figure 7. Six isolated compounds from chloroform fraction

The yellowish colored compound gave single spot in TLC with chloroform: ethyl acetate (9:1) eluent system but it contains some impurities. It was re-purified with preparative thin layer chromatography with solvent system chloroform: ethyl acetate (9:1). After re-purifying the yellowish colored compound in preparative thin layer chromatography it gave clearly separated and visible yellowish component. This component was collected in 50 ml flask and the colored compounds was dissolved with acetone and filtered with whatmann filter paper No. 1.

The re-purified yellowish colored compound is shown in the following figure.

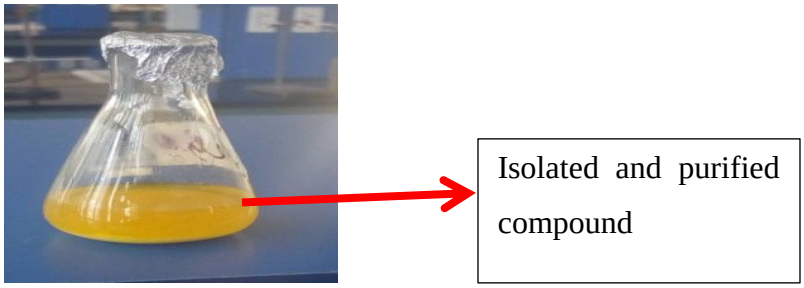


Figure 8. Yellowish colored compound of chloroform fraction

The filtered yellowish colored compound was kept in deep refrigerator at -20 ^{0}c. After 4 days the acetone dissolved yellowish colored compound was concentrated in rotary evaporator under reduced pressure. Only yellowish oil colored film was left in the florentine flask and washed with small amount of acetone, and using clean capillary tube, the yellowish colored compound was transferred in to sample vials for spectroscopic analysis.

4.5. Antimicrobial Activity Procedure

One gram positive bacterial species (Staphylococcus aurous, clinically isolated) and one gram negative bacterial species (Escherichia coli, clinically isolated) were used for this study. All bacterial species were obtained from college medicine and of health science, University of Gondar**, Ethiopia** and collected in the department of biotechnology, University of Gondar. They were maintained on Mueller Hinton Agar at 4^0c prior to use. The methanol crude extract of *zehneria scabra* leaves fraction (petroleum ether fraction, chloroform fraction, ethyl acetate and methanol fraction) were screened for their in-vitro antibacterial activity in comparison with standard antibiotic vancomycin (1oomg/ml) by using disc diffusion method. In the study of the antimicrobial activities, concentrations of 100mg/ml of each extract were, used for screening. This was done by dissolving 1.0g of each crude extract in 2ml of acetone. Each of solid extracts was reconstituted in acetone solvent to obtain a stock solution of 100mg/ml. Medium was prepared by dissolving 11.4g of Mueller Hinton Agar in 300ml of distilled water and heated on hot plate, and then autoclaved and dispensed at 20ml per plate in 12x12 petridishes. Set plates were incubated over night to ensure sterility. After that, microorganisms were added in to medium and each labeled medium plate was uniformly inoculated with a test organism by using a sterile cotton swab rolled in the suspension to streak the medium plate surface in a form that lowness growth can be observed. A sterile cork borer of 5mm diameter was used to make wells on the medium. 100mg/ml of the methanol crude extract fractions were dropped in to each appropriate labeled well. Mueller Hinton Agar medium plates were incubated at 37^0c for 24 hours. Antimicrobial activity was determined by measuring the diameter of zones of inhibition (mm) produced after incubation.

4.6. Preliminary Phytochemical Test: Phytochemical Screening

Phytochemical screening was carried out on the crude extract of 99% CH_3OH as solvent using standard procedures to identify the constituents as described [20] for secondary metabolites such as alkaloids, flavonoids, phenols, terpenoids, tannins, saponins, carbohydrate and glycosides.

4.6.1. Test for Alkaloids

Mayer's reagent: To 2N HCl was added in to 2ml of the crude extract in the test tube. The mixture was heated for 20 minutes, cooled and filtered. Then 1ml of filtrate was tested with Mayer's reagents. Formation of cream precipitate for Mayer's indicated presence of alkaloids [30].

4.6.2. Test for Flavonoids

Shinoda test: pieces of magnesium ribbon and concentrated HCl was mixed with crude plant extract and after a few minutes pink colored scarlet appeared which indicated the presence of flavonoids.

Alkaline reagent test: 2 ml of 2% NaOH solution was mixed with plant crude extract, intensive yellow color was formed, which turned in to colorless when 2 drops of diluted acid was added to the solution, this result indicated the presence of flavonoids [31].

4.6.3. Test for Terpenoids

Salkowski test: 2 ml of the crude extract was dissolved in 2 ml of chloroform and evaporated to dryness. 2 ml of Conc. H_2SO_4 was then added and heated for about 2 minutes. Development of a grayish color indicated the presence of terpenoids [32].

4.6. 4. Test for Glycosides

Liebermann's test: 2 ml of acetic acid and 2 ml of chloroform were mixed with 2ml of crude extract. The mixture was then cooled and concentrated H_2SO_4 was added. Green color indicated the entity of aglycone steroidal part of glycosides [12].

Salkowski's test: about 2 ml of concentrated H_2SO_4 was added to the 2ml of crude extract. A reddish brown color produced indicating the entity of steroidal aglycone part of the glycoside. 2ml of crude extract solution, equal quantity of Fehling's solution was added and the solution was heated for a few minutes. A brick red precipitate indicated the presence of glycosides [30].

4. 6. 5. Test for Phenols

2ml of 2% solution of $FeCl_3$ was mixed with crude extract. Black or blue-green color indicated the presence of phenols [31].

4.6.6. Test for Carbohydrates

500 mg of powdered sample was taken and dissolved in 5 ml of distilled water and then filtered. Filtrate was added with few drops of Molisch's reagent, followed by addition of 1 ml of concentrated H_2SO_4 by the side of the test tube. After two minutes, 5 ml of distilled water was added. Red or dull violet color formation at the interphase of the two layers was taken as positive test [33].

4.6.7. Test for Tannins

About 2 ml of the crude extract was stirred with 2ml of distilled water and few drops of ferric chloride ($FeCl_3$) solution were added to the solution. Formation of green precipitate indicated the presence of tannins [32].

4. 6.8. Test for Saponins

Foam test: 10 g of powdered sample was boiled in 10 ml of distilled water and then filtered. 3 ml of distilled water was added to filtrate and shaken vigorously for about 5 min. Formation of foam after shaking was taken as a confirmation for the presence of saponins [33].

5. RESULTS AND DISCUSSION

5.1. Result of Phytochemical Screening

The results of phytochemical analysis of methanol leaves extract of *zehneria scabra* were tabulated in table 1. Phytochemical studies revealed that the presence of alkaloids, flavonoids, glycosides, terpenoids, phenols and tannins in the methanol extract of *zehneria scabra* leaves. Saponins and carbohydrates were absent in the methanol crude extract of *zehneria scabra* leaves.

Phytochemical screening of methanol crude extract of *zehneria scabra* leaves are summarized in table 1.

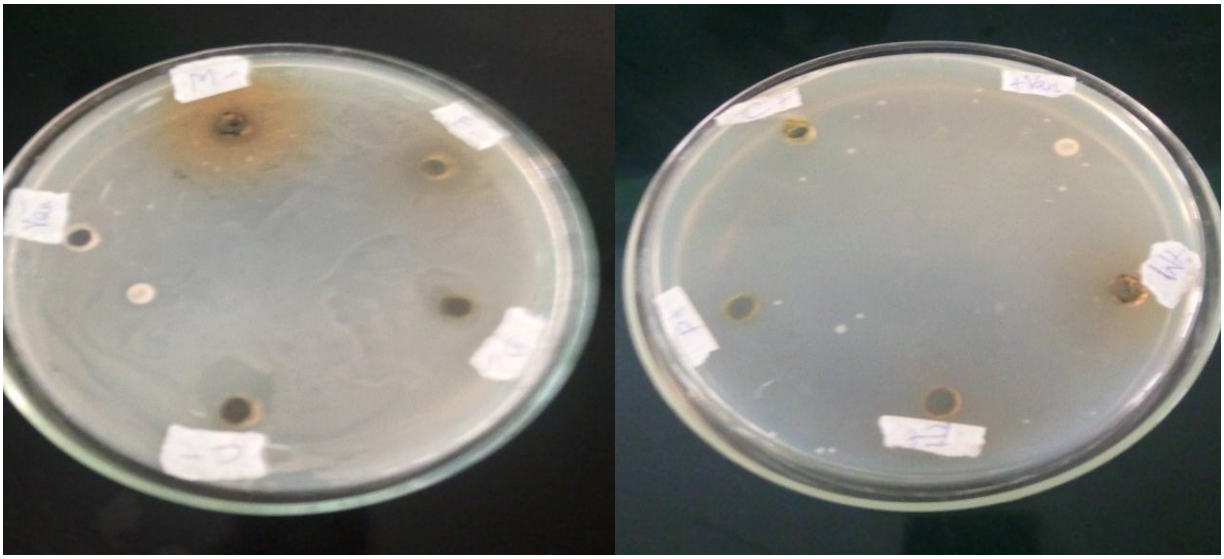
Phytochemical	Test	Test result
Alkaloids	Mayer's test	+
Flavonoids	Shinoda test	+
	Alkaline reagent test	
Terpenoids	Salkowski test	+
Glycosides	Libermann's test	+
Phenols	Ferric chloride test	+
Carbohydrates	Molisch's test	-
Tannins	Ferric chloride test	+
Saponins	Foam test	-

Note: + indicates present and - indicates absent

Table 1. Phytochemical screening of methanol crude extract of *zehneria scabra* leaves

5.2. Result for Antimicrobial Activity

Antimicrobial assay was done based on disc diffusion method and the crude extract fraction for all solvent system shown by the following petridish.



Gram negative Gram positive

Note: M indicates methanol fraction, E indicates ethyl acetate fraction, C indicates chloroform fraction and P indicates petroleum ether fraction.

Figure 9. Inhibition Zone of E. coli and S. Aureus Bacterial Species at 100mg/ml concentration

5.2.1. The methanol fraction of crude extract of *zehneria scabra* leaves were tested for antimicrobial assay.

The inhibition zones of methanol fraction of crude extract at 100mg/ml concentration against some common bacteria and the result is summarized in table 2 below.

Bacteria species		Zone of inhibition (mm)	
		Zehneria Scabra	
Gram Positive	S. Aureus	100mg/ml	Vancomycin
		10	8
Gram Negative	E. Coli	15	10

Table 2. The inhibition zone of methanol fraction at 100mg/ml against some common bacteria.

5.2.2. Antibacterial test for the ethyl acetate fraction of methanol crude extract.

Bacteria species		Zone of inhibition (mm)	
		Zehneria Scabra	
Gram Positive	S. Aureus	100mg/ml	Vancomycin
		15	8
Gram Negative	E. Coli	20	10

Table 3.The inhibition zone of ethyl acetate fraction of methanol crude extract

5.2.3. The antimicrobial test for chloroform fraction of methanol crude extract

The antimicrobial test for chloroform fraction is given in the following table 4.

Bacteria species		Zone of inhibition (mm)	
		Zehneria Scabra	
Gram Positive	S. Aureus	100mg/ml	Vancomycin
		14	8
Gram Negative	E. Coli	21	10

Table 4. The inhibition zone of chloroform fraction of methanol crude extract

5.2.4. Antimicrobial test for petroleum ether fraction of methanol crude extract

The antimicrobial test for petroleum ether is given in the following table 5.

Bacteria species		Zone of inhibition (mm)	
		Zehneria Scabra	
Gram Positive	S. Aureus	100mg/ml	Vancomycin
		8	8
Gram Negative	E. Coli	17.5	10

Table 5. Inhibition zone of petroleum ether fraction of methanol crude extract.

The methanol crude extract fraction of *zehneria scabra* leaves was investigated for their in vitro antibacterial activities. The above table shows that methanol crude extract fraction of various solvent system (petroleum ether, chloroform, ethyl acetate and methanol fraction) inhibited both gram negative (Escherichia Coli) and gram positive (S. aureus) bacterial species. The chloroform and ethyl acetate methanol crude extract fraction at standard concentration (100mg/ml) result shows more potent to inhibit the growth of bacterial species. Specially, the chloroform and ethyl acetate fraction of methanol crude extract inhibited gram negative bacteria (E. Coli) at 100mg/ml concentration. In other study, chloroform fraction and ethyl acetate fraction of methanol crude extract of *zehneria scabra* leave showed growth inhibition against S. aureus and E.coli [20]. The petroleum ether fraction result shows medium potent to inhibited gram negative bacteria at 100mg/ml concentration and methanol crude extract fraction result shows minimum inhibition at 100mg/ml concentration for gram negative (E.Coli) bacterial species and also, in methanol fraction and petroleum ether fraction of methanol crude extract result shows minimum inhibition for gram positive bacteria (S. aureus) at 100mg/ml concentration.

5.3. Compound Characterization

5.3.1. Inferences from Infrared (FT-IR) spectrum

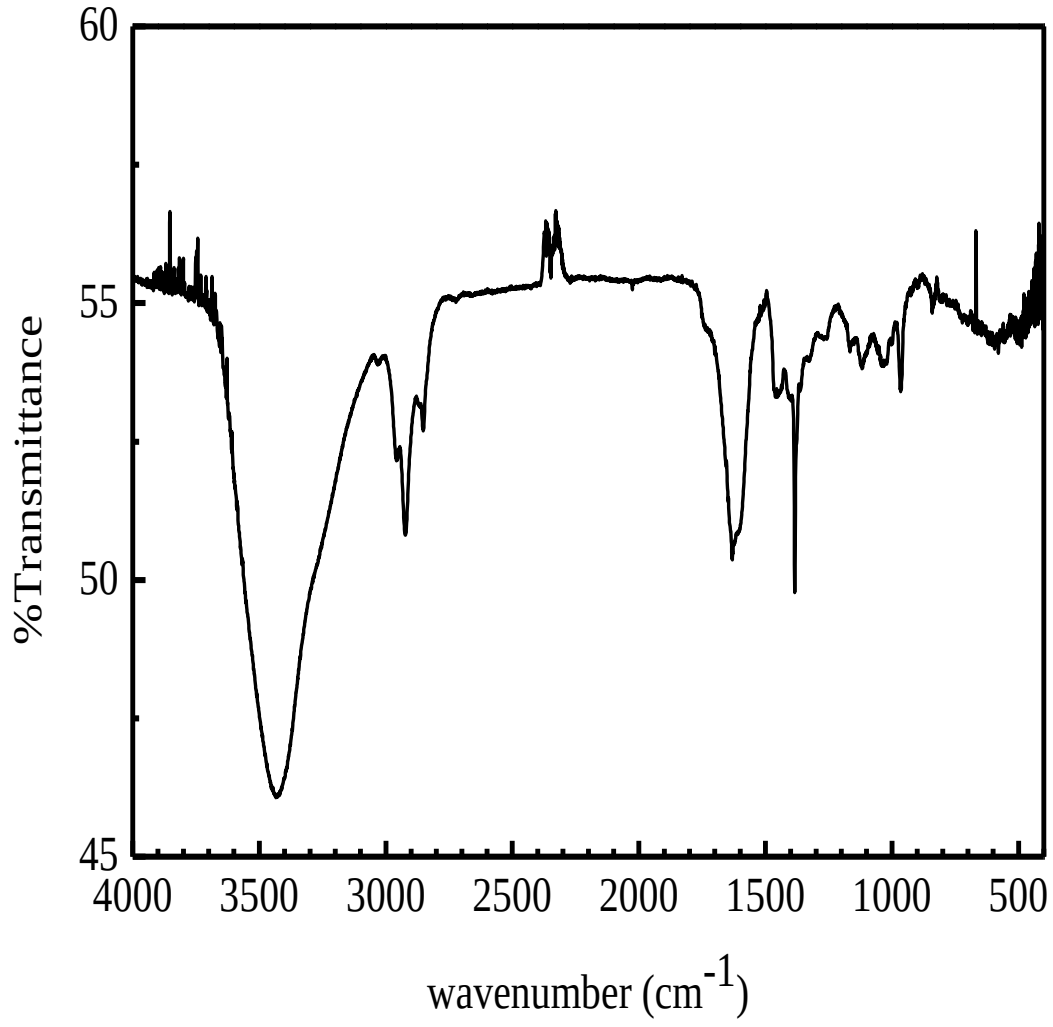


Figure 10. IR spectra of yellowish compound

Preparative thin layer chromatographic purification and isolation of the leave extract of *zehneria scabra* afforded yellowish oil compound isolated for the first time from this genera. Compound was obtained as yellowish oil (25mg) with Rf value of 0.39 (10% ethyl acetate in 90% chloroform) solvent system. The IR spectrum exhibited absorptions at ≈ 3455 cm^{-1}indicated presence of hydroxyl group (-OH), ≈ 3090 cm^{-1} indicated presence of =C-H stretching, ≈ 2946 cm^{-1} and ≈ 2840 cm^{-1} indicated presence of –C-H stretching vibrations, ≈ 1690 cm^{-1} indicated presence of carbonyl group and also, ≈ 1420 cm^{-1} due to presence of C-C vibrations, ≈ 1000 cm^{-1} indicated presence of C-O vibrations and information from IR spectrum is given in the following table 6.

No.	IR Absorption region (cm^{-1})	Groups
1	≈ 3455	-OH
2	≈ 3090	=C-H
3	≈ 2946 and 2840	-C-H
4	≈ 1690	C=O
5	≈ 1420	C-C
6	≈ 1000	C-O

Table 6. IR Spectra of yellowish compound

5.3.2. Inferences from ^{1}H NMR Spectrum

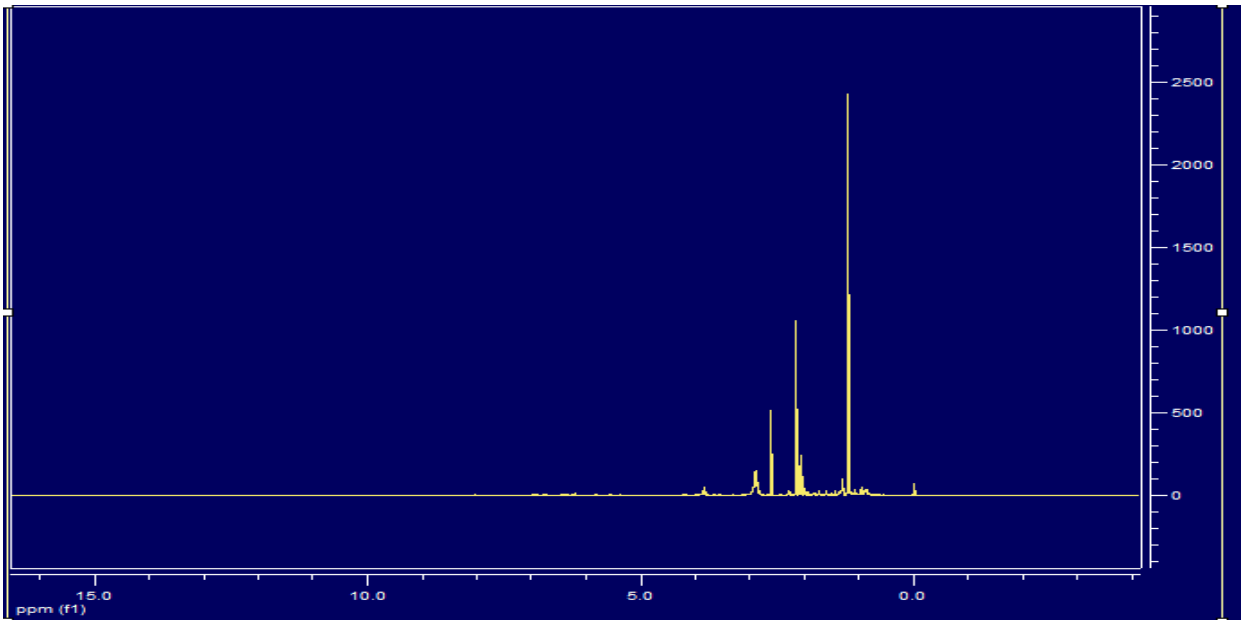
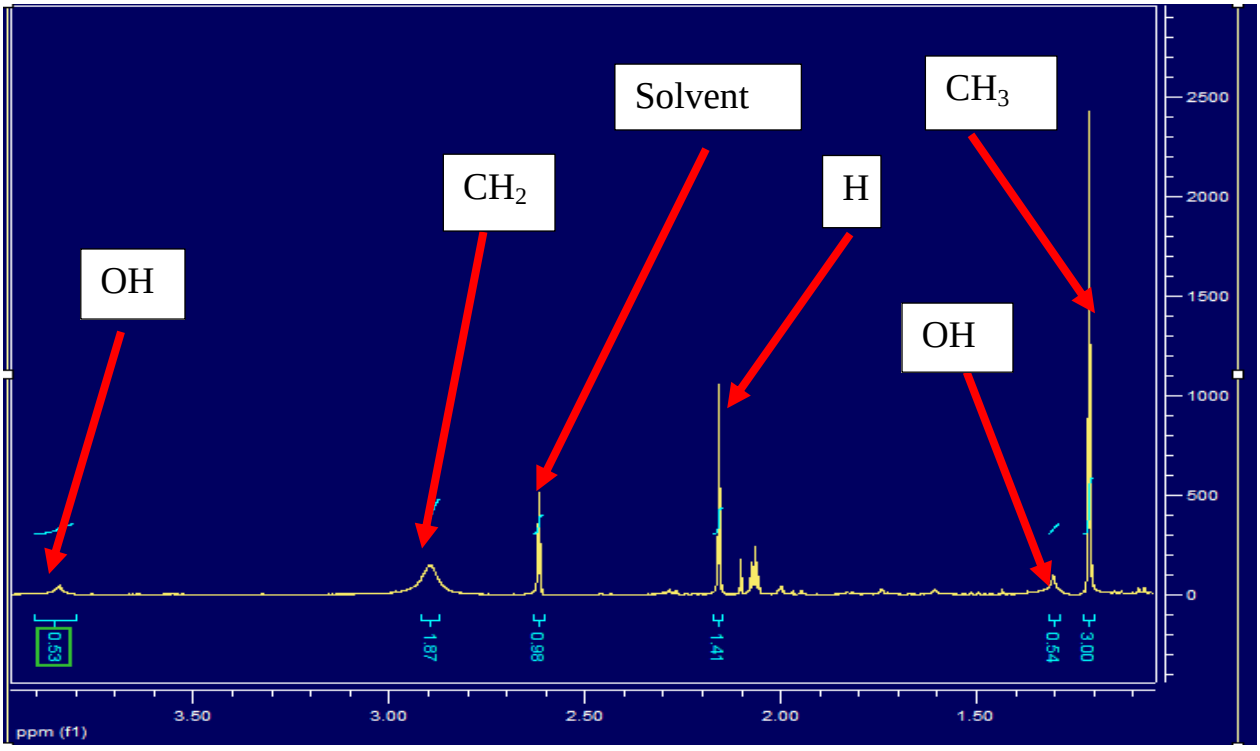
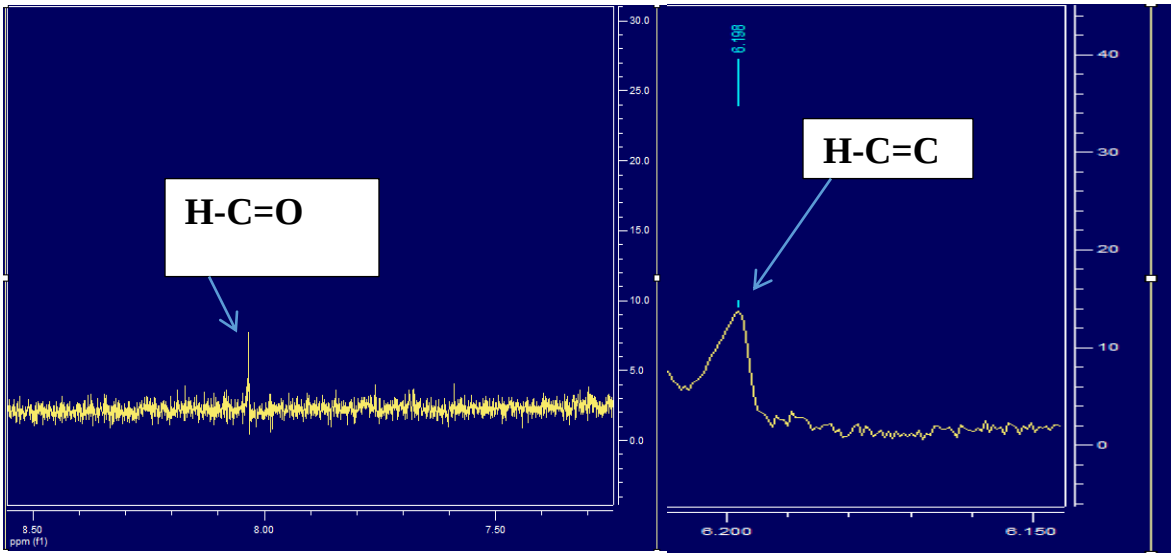


Figure 11. 1H NMR Spectra of yellowish compound.

The interpreted ^{1}H NMR Spectra is given in the following spectrum





5.3.3. Inferences from ^{13}C NMR Spectrum

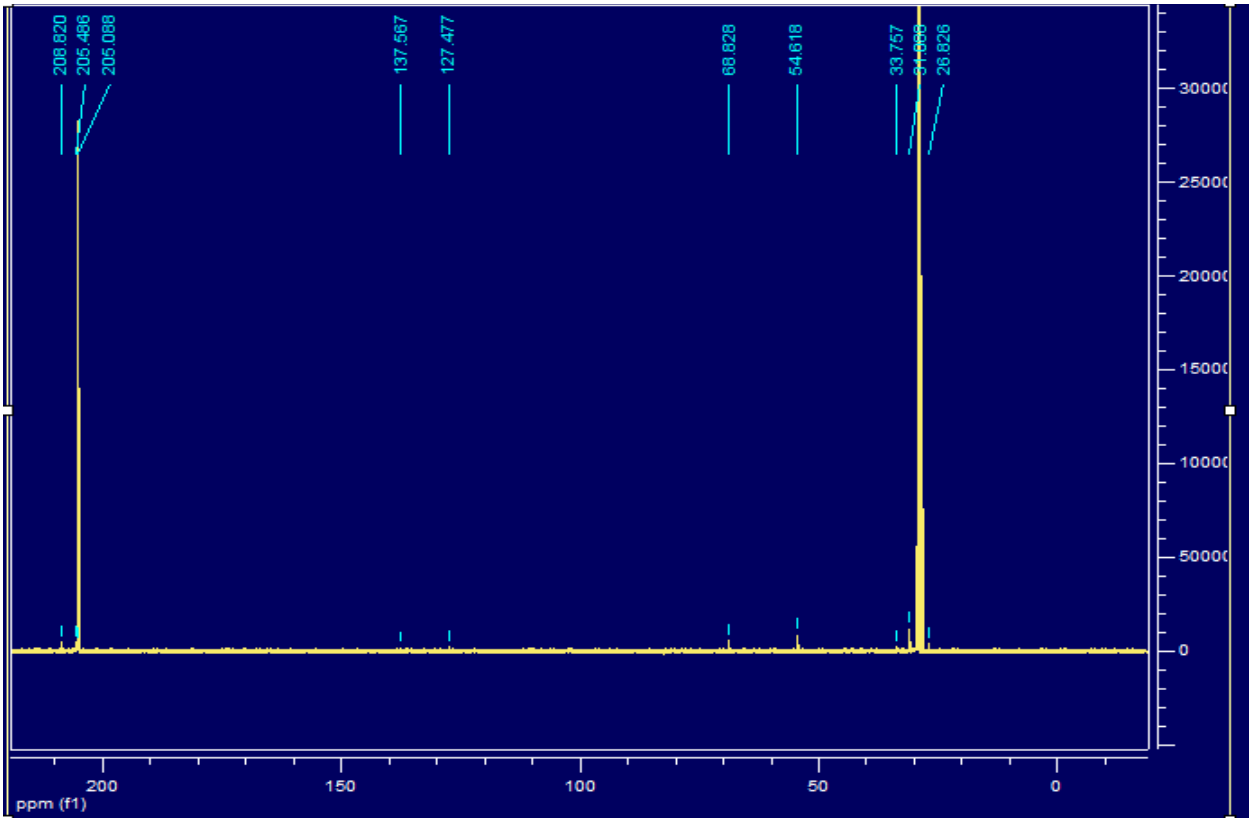
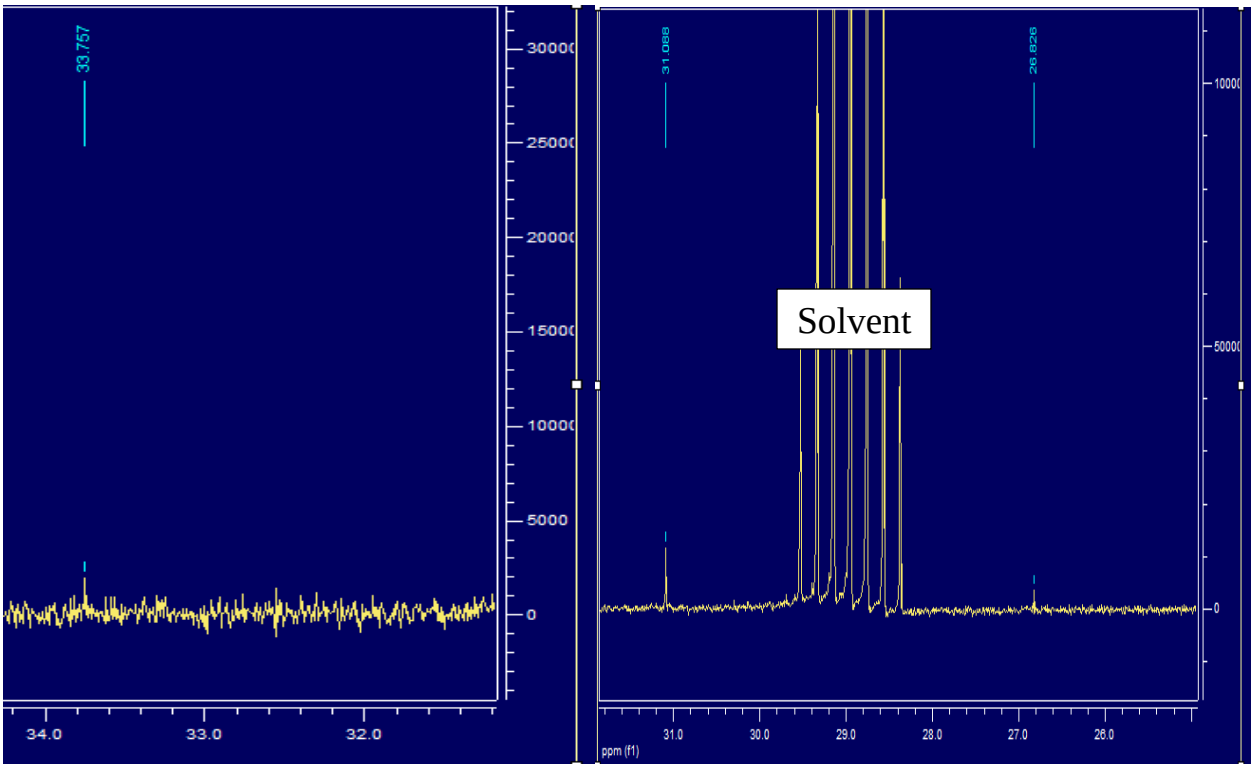
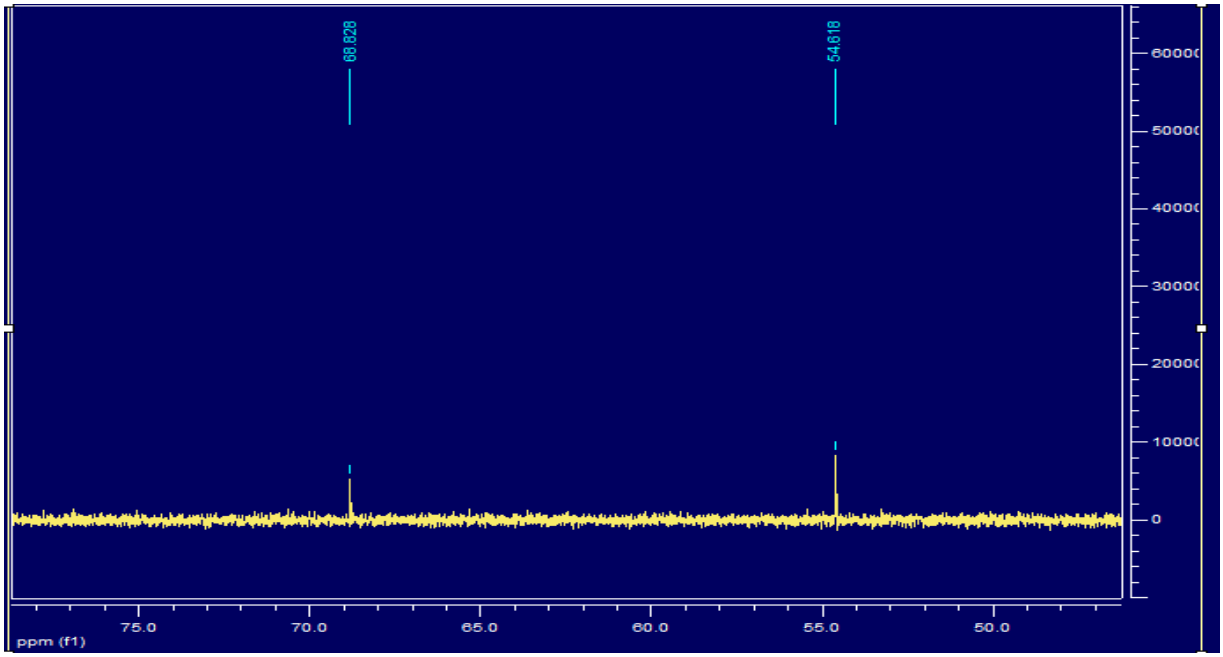
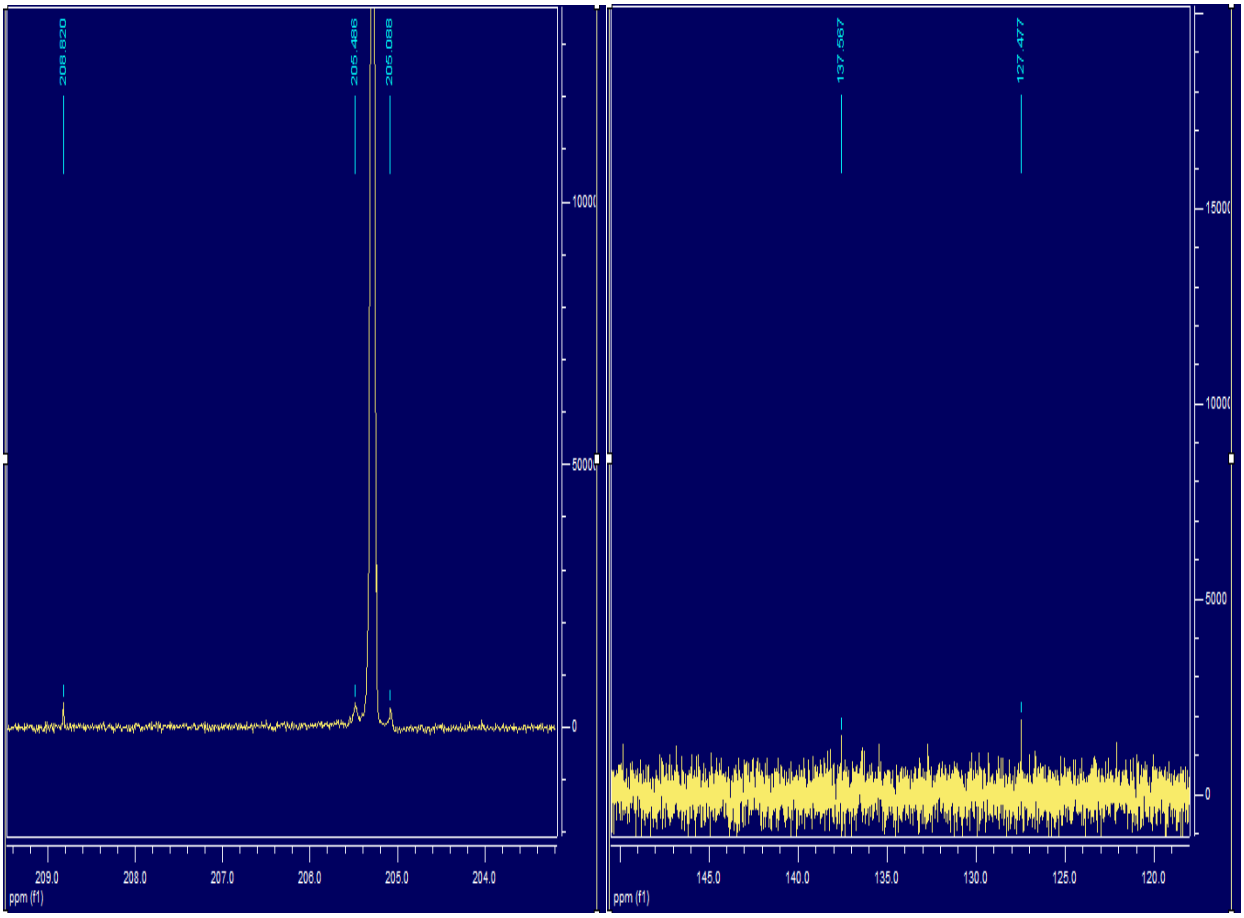


Figure 12. ^{13}C NMR Spectra of yellowish compound

The interpreted ^{13}C NMR Spectra is shown below






208.820
205.486
205.088
10000
5000
0
209.0
208.0
207.0
206.0
205.0
204.0
ppm (f1)
137.567
127.477
15000
10000
5000
0
145.0
140.0
135.0
130.0
125.0
120.0
ppm (f1)

5.3.4. Inferences from DEPT Spectrum

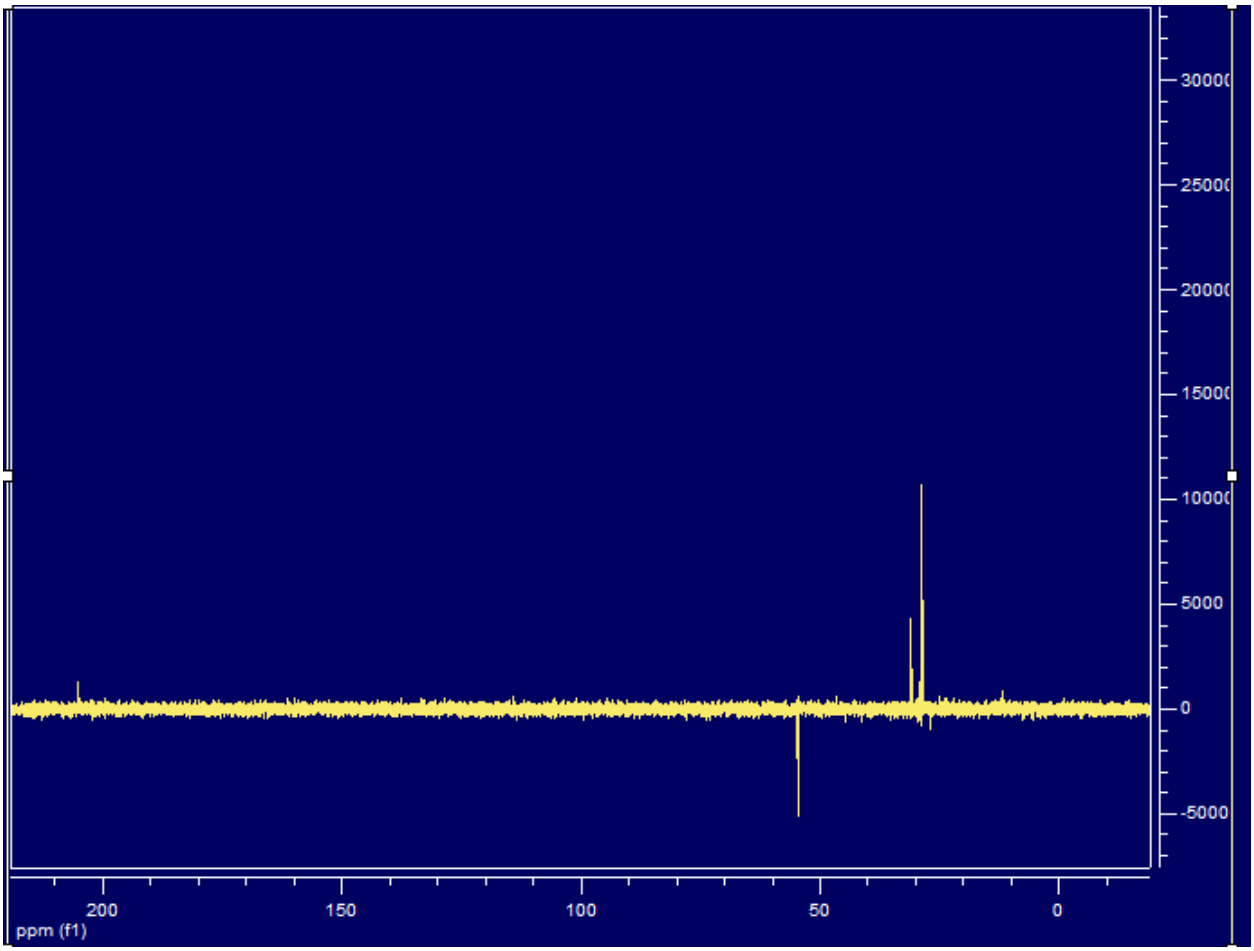


Figure 13. DEPT Spectra of yellowish compound

The ^{1}H NMR Spectrum (Acetone-$d_{6,}$ Table 7) revealed that the existence of aliphatic protons, hydroxyl protons, olefin protons (sp^2 hybridized carbon) and aldehyde protons. The two hydroxyl protons resonating at δ3.84 and δ 1.303 which are fixed at position of C-1' and C-2' respectively, the aliphatic protons resonating at [δ2.16 ($H_{, S}$), δ2.90 (2H, s) and δ1.21 (3H, s)]. The olefin protons (sp^2 hybridized carbon proton) resonating at δ6.20 (H, s) and aldehyde proton resonating at δ8.04 (H, s). The ^{13}C NMR spectrum for yellowish compound showed 10 carbon signals of which two olefin or sp^2 hybridized carbon atom resonating at δ127.48 and δ137.57 which are assigned to position C-2 and C-3 respectively. The ^{13}C NMR also indicated the presence of two quaternary non-protonated carbon atom at δ33.76 and δ68.83 consistent with

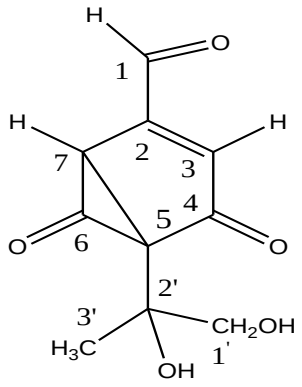
DEFT-135 Spectra and assigned as C-5 and C-2' respectively. Furthermore, the ^{13}C NMR spectrum also revealed the presence of three sp^3 hybridized (aliphatic) carbons, resonating at [δ26.83, δ31.00 and δ54.62] and three carbonyl groups resonating at δ205.49, δ205.09 and δ208.82 consistent with DEFT-135 Spectra and assigned as C-1, C-4 and C-6 respectively. The DEPT-135 spectrum reveals the presence of one CH_3 resonating peaks at δ26.83 (CH and CH_3 gives positive peaks in DEPT-135 Spectrum whereas CH_2 gives negative peaks in DEPT spectrum and there is no peak for quaternary carbons in DEPT spectrum). The one CH_2 group (aliphatic carbon) is resonating at δ54.62 and also, one CH resonating at δ31.00. The aldehyde proton resonating at δ205.49 in DEPT-135 Spectrum.

The complete ^1H NMR, ^{13}C NMR and DEPT-135 Spectra of yellowish compound in acetone-d_6, data is given in the following table.

C/ H atom	Multi	δC (ppm)	δH (ppm)
C-1	CH	205.49	8.04 (s)
C-2	C	137.57	-
C-3	CH	127.48	6.20 (s)
C-4	CO	205.09	-
C-5	C	33.76	-
C-6	CO	208.82	-
C-7	CH	31.00	2.16 (s)
C-1'	CH_2	54.62	2.90 (s)
C-2'	C	68.83	-
C-3'	CH_3	26.83	1.21 (s)
1'-OH	-OH	1'-OH	1.303 (s)
2'-OH	-OH	2'-OH	3.84 (s)

Table 7. Complete 1D NMR spectra of yellowish compound in acetone-d_6

From extensive interpretation of IR, ^{1}H NMR, ^{13}C NMR and DEPT-135 Spectrum the possible structure of yellowish compound is given as follows.



5-(1', 2'-dihydroxy propan-2'-yl)-4-6-dioxobicyclo [3.1.0] hex-2-ene-2-carbaldehyde

Figure 14. Possible structure of characterized compound

6. CONCLUSION

The researcher made the first attempt to investigate phytochemical constituents of organic leave extract of *zehneria scabra* from Ethiopian origin. Phytochemical screening of the leave extract of *zehneria scabra* revealed that the presence of alkaloids, flavonoids, terpenoids, glycosides. Phenols, tannins and indicated the absence of carbohydrate and saponins. The antimicrobial activity test for methanol crude extract fraction in various solvent systems revealed that chloroform and ethyl acetate fraction is more potent to inhibit the growth of gram negative and gram positive bacterial species. The petroleum ether fraction result shows medium inhibition and methanol fraction result shows minimum inhibition for gram negative bacteria and also, methanol and petroleum ether fraction result shows that minimum inhibition for gram positive bacterial species. Chromatographic separation of the chloroform: ethyl acetate (9:1) yielded monoterpenoid compound.

Monoterpenoids are one of the classes of terpenoids and are common in many plant species. And are used in cosmetic, non-cosmetic and pharmaceutical preparations, as well as in food industry. They exhibit diverse pharmacological effects and also, exhibit effect of relaxation on ileum smooth muscle. In order to make these compounds applicable for clinical use, they should be studied more extensively to understand their bioavailability, metabolic pathways and toxicity in humans. This study contributes in shading light on pharmacologically important secondary terpenoids metabolites from leave extracts with vast therapeutic uses and the ethno medicinal value of the plant. Further work is recommended on the stem, root and fruit extracts of the plant so as to identify more novel and bioactive compounds in support of its traditional use.

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